



Trefoil factor peptide 3 is positively correlated with the viscoelastic properties of the cervical mucus plug

Bastholm, Sara Kjær; Samson, Mie Hesselund; Becher, Naja; Hansen, Lea Kirstine; Stubbe, Peter Reimer; Chronakis, Ioannis S.; Nexø, Ebba; Uldbjerg, Niels

Published in:
Acta Obstetricia et Gynecologica Scandinavica

Link to article, DOI:
[10.1111/aogs.13038](https://doi.org/10.1111/aogs.13038)

Publication date:
2017

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Bastholm, S. K., Samson, M. H., Becher, N., Hansen, L. K., Stubbe, P. R., Chronakis, I. S., Nexø, E., & Uldbjerg, N. (2017). Trefoil factor peptide 3 is positively correlated with the viscoelastic properties of the cervical mucus plug. *Acta Obstetricia et Gynecologica Scandinavica*, 96(1), 47-52. <https://doi.org/10.1111/aogs.13038>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Received Date : 28-May-2016

Revised Date : 08-Sep-2016

Accepted Date : 07-Oct-2016

Article type : Original Research Article

Trefoil factor peptide 3 is positively correlated with the viscoelastic properties of the cervical mucus plug

Running headline: Cervical mucus plug, viscoelasticity, TFF3

Sara Kjær BASTHOLM¹, Mie Hesselund SAMSON², Naja BECHER³, Lea Kirstine HANSEN¹, Peter Reimer STUBBE⁴, Ioannis S. CHRONAKIS⁵, Ebba NEXO² & Niels ULDBJERG¹

¹Department of Obstetrics and Gynecology, Aarhus University Hospital, Aarhus,

²Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, ³Department of Clinical Genetics, Aarhus University Hospital, Aarhus, ⁴Food Production Engineering Research Group, Technical University of Denmark, Kgs. Lyngby, ⁵Nano-BioScience Research Group, Technical University of Denmark, Kgs. Lyngby, Denmark

Correspondence:

Niels Ulbjerg

Department of Obstetrics and Gynecology, Aarhus University Hospital, Palle Juul-Jensens, Boulevard 99, 8200 Aarhus N, Denmark

E-mail: uldbjerg@clin.au.dk

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/aogs.13038

This article is protected by copyright. All rights reserved.

Conflicts of interest statement:

All authors explicitly declare no conflicts of interest.

Abstract

Introduction. The viscoelastic properties of the cervical mucus plug are considered essential for the occlusion of the cervical canal and thereby for protection against ascending infections during pregnancy. Factors controlling this property are virtually unknown. This study explores a possible role of trefoil factor peptides 1, 2 and 3 (TFF1-3); peptides believed to influence mucus viscosity. *Materials and methods.* The study is based on spontaneously shed cervical mucus plugs from 14 women in active labor. The viscoelastic properties; the elastic modulus (G') and the viscous modulus (G'') were determined by an oscillatory rheometer. The concentrations of TFF1-3 were measured by an in-house ELISA. Associations were analyzed by random-effects generalized least squares regression analyses. *Results.* Median (range) concentrations of TFF1, (TFF2) and [TFF3] were 3.1 (1.2-8.6), (1.1 (<0.006-3.7)) and [1000 (170-5300)] nmol/g cervical mucus plug. The TFF3 concentration was associated with G' (regression coefficient 11.7 Pa/ Log nM (95 % CI; 3.0 – 20.4, $p = 0.009$) and G'' (regression coefficient 3.2 Pa / Log nM (95% CI; 1.5 – 5.0, $p < 0.001$). *Conclusion.* We suggest that TFF3 plays a role for the viscoelastic properties of cervical mucus plug.

Key words:

pregnancy, cervical mucus plug, viscoelasticity, rheology, trefoil factor peptides1-3.

Abbreviations:

CMP cervical mucus plug

TFF1 trefoil factor peptide 1

TFF2 trefoil factor peptide 2

TFF3 trefoil factor peptide 3

G' Elastic Modulus
G'' Viscous Modulus

Key Message:

Trefoil factor peptides (TFF1-3) are present in cervical mucus plugs at term and TFF3 is positively correlated with the viscoelastic properties of the plug.

Introduction

During pregnancy, the cervical mucus plug (CMP) occludes the cervical canal and forms a barrier between the more or less sterile uterine cavity and the microbe-rich vagina (1). High concentrations of antimicrobial and antifungal peptides, immune cells and immunoglobulins are essential for this function (2-4). This immunological barrier function is probably sustained by the viscoelastic properties of the CMP, which ensures that the plug remains in the cervical canal despite daily activities including intercourse and even when the cervical canal at the end of pregnancy is dilated to more than one or two cm. The passage of pathogens might also depend on the elastic and viscous properties (5) of the CMP as does the passage of spermatozoa through cervical mucus from non-pregnant women (6). The factors controlling the elastic and viscous properties of the CMP remain virtually unknown.

The Trefoil factor peptides (TFFs) constitute a family of small (12-22 kDa) molecules containing one (TFF1 and TFF3) or two (TFF2) trefoil domains(7). TFFs are most abundantly expressed in the gastrointestinal tract, yet they can be detected in virtually all tissues containing mucin-secreting cells including the endocervix and the uterus(7;8). Despite intensive investigations the exact functions of the TFFs are largely unknown. They have been suggested to cross-link mucin hereby forming stable gel complexes which are resistant to gastrointestinal proteases, a broad range of noxious agents, and mechanical stress(9;10). In vitro studies have shown that TFF2 significantly increases the viscoelastic properties of porcine stomach mucin when added in supra-physiological concentrations (11). Furthermore significant increases in the viscosity of gastric mucus from mice were

observed when TFF2 was injected intravenously or subcutaneously (12). It is, however, still unknown whether TFFs in physiological concentrations influence the rheological properties of mucus.

We aimed to investigate the presence of TFFs in CMPs and to study a possible association between CMP viscoelasticity and the concentrations of TFF1-3 measured in CMPs shed during active labor at term.

Material and methods

Initially, CMPs were collected from 16 women in active labor (cervical dilation 4 to 10 cm) at the delivery wards of Aarhus University Hospital, Denmark. The median (range) age of the women included was 29.5 (21-36) years, the median gestational age was 40⁺¹ weeks (38⁺⁰ – 42⁺⁰ weeks) and median parity was 1.5 (1 – 4). The CMPs were shed spontaneously or manually retrieved during vaginal exploration. All 16 CMPs were cut into specimens of approximately 1.4 g. Two to seven specimens were obtained from each CMP depending on the original size, resulting in a total of 42 specimens. From each of these specimens, a 0.1 g biopsy was frozen and stored at -80° C for later biochemical analysis (TFF1-3 and hemoglobin concentration). The specimens (median (range) weight 1.37 g (1.23 – 1.46 g)) were frozen at -80° C for at least 24 hours and thawed on ice prior to rheological analysis. Nine of the 42 specimens were excluded as the hemoglobin concentration of the biopsy exceeded 200 µM. Thus, the results presented are based on 33 specimens representing 14 CMPs.

The method for assessing rheological properties of the CMP including determining the linear viscoelastic region has previously been described(13). Briefly, the rheological properties were assessed by a dynamic oscillatory rheometer (Stress Tech, Reologica instruments AB, Lund, Sweden) within the linear viscoelastic region and at 37.0 °C. Each of the 33 specimens were firstly subjected to a frequency sweep where specimens were

deformed with a fixed stress (5 Pa) at increasing frequency (0.01 – 1 Hz) (Figure 1A). Secondly, the specimens were subjected to a stress sweep where specimens were deformed with an increasing stress (1-300 Pa) at a fixed frequency of 1 Hz (Figure 1B). A number of rheological variables were obtained from these sweeps. The *elastic modulus* (G') reflects the elastic-like component of viscoelastic behavior, and the *viscous modulus* (G'') reflects the liquid-like component (14-17). Furthermore, in stress sweeps the cross-over point of G' and G'' which denotes the amount of stress required to deform the internal CMP microstructure to the point where effective flowing occurs ($G'' > G'$) was obtained.

The CMP specimen-biopsies were extracted as described previously (18). The 0.1 g specimen-biopsy was diluted 1:30 in a buffer containing 50 mM Tris-hydrochlorate, 10 mM CaCl_2 , 0.05% BrijTM35 and 1 mM Phenylmethylsulfonyl fluoride (pH 7.4) during manual homogenization. An aliquot of this 1:30 dilution was then extracted overnight with rotation at 4°C. After centrifugation (20 minutes, 16,000 g) the supernatant was removed (*first extraction*) and placed on ice. Depending on the volume of the first extraction, an adjusted amount of buffer was added; the pellet was re-homogenized, re-extracted overnight and subjected to centrifugation. Again, the supernatant was removed (*second extraction*), pooled on ice with the first extraction and kept on ice. The extraction process was completed by a short heat extraction (4 min, 60°C, *third extraction*) during which the final 1:100 dilution was reached. Extractions were kept at -80°C until analysis. The TFF1, TFF2 and TFF3 concentrations were assessed on the 1:100 extractions after further 100 fold dilution employing in house ELISA methods (19;20). Furthermore, hemoglobin concentrations in the extracts were determined with the routine method of the laboratory (Cobas6000, Roche A/S Hvidovre, Copenhagen, Denmark - <http://www.roche.dk/home/produkter.html>).

Statistical analyses were performed in STATA IC12 (StataCorp LP, College Station, TX, USA). The variations of TFF1-3 concentrations within and between CMPs were investigated using mixed-effects restricted maximum likelihood regression analysis.

To evaluate the association between rheological parameters and TFF1-3 concentrations, regression coefficients were estimated using random-effects generalized least squares regression analysis with and without adjustment for specimen weight. Two-sided p -values below 0.05 were considered significant. TFF measurements were log-transformed to obtain a normal distribution and an equal variance. All statistical analyses take into account the clustering of the data.

The Central Denmark Region Committees on Biomedical Research Ethics approved the project (project number M-ÅA-20050053, amendment 26296) according to declaration of Helsinki. The study is registered in the Danish data protection agency (project number 1-16-02-175-12).

Results

TFF1 and TFF3 were detected in all 14 CMPs and TFF2 in 8 of the 14 CMPs. The most abundant TFF was TFF3 with a median (range) concentration of 1000 (170-5300) nmol/g CMP as compared to 3.1 (1.2 – 8.6) nmol/g CMP for TFF1 and 1.1 (< 0.006 – 3.7) nmol/g CMP for TFF2 (Figure 2).

All CMP specimens showed solid-like viscoelastic behaviour with predominating elastic properties, as seen in the representative example shown in Figure 1A-B. We found that the elastic modulus (G') was about three to four times greater than the viscous modulus (G'') at all frequencies (13). Likelihood ratio tests evaluated to what extent the TFF variation between the specimens reflected differences between CMPs or differences within the CMPs, i.e. heterogeneity. The differences between CMPs were highly significant concerning all three TFFs, TFF1 and TFF3 ($p < 0.001$) and TFF2 ($p = 0.002$). This was not only reflected by the p -values, but also by the high point estimates and relatively narrow confidence interval for the intra class correlation coefficients values (Table 1). The viscoelastic properties of the CMPs were positively correlated with the TFF3 concentration. The CMP specimens ($n = 33$) with higher levels of TFF3 showed corresponding higher

values in their elastic and viscous modulus (Table 2 and Figure 3A-D) and the associations remained constant when adjusting for specimen weight.

TFF3 was significantly associated with the viscoelastic properties at all frequencies investigated (0.01 - 1Hz) in the frequency sweep, however, the association was less pronounced at the higher frequencies (1 Hz compared to 0.01 Hz). TFF3 was associated with the viscoelastic properties obtained by stress sweeps (0-300Pa), however, this association were also less pronounced at higher stresses (15 Pa compared to 1 Pa) and not significant beyond the linear viscoelastic region (1-15Pa) (Table 2). Furthermore, TFF3 was not associated with the cross-over point obtained by stress sweep (Table 2). For TFF1 and TFF2 associations with CMP viscoelastic properties were much less pronounced and not significant ($p > 0.15$). Specimens with non-detectable levels of TFF2 were appraised to 3 pmol/g CMP (assay detection limit of 6 pmol/ g CMP), in order to carry out the regression.

Discussion

This study documents the presence of TFF1, TFF2 and TFF3 in CMPs obtained at term of pregnancy. Furthermore, rheological analysis demonstrates that the elastic as well as the viscous properties of the CMPs increase with increasing TFF3 concentrations.

The main strength of the study is that all rheological measurements were obtained by an established and validated method controlling the experimental duration, temperature, specimen size, and the linear viscoelastic region (13). Most probably, the blood contamination did not affect the rheological results as specimens with hemoglobin concentrations above 200 μ M were excluded (13). Neither, did the blood affect the assessments of the TFF3 levels of the CMPs, as serum levels are only about 5.0 nM (21) as compared to the median level of 1000 nmol/g CMP.

We have shown that TFF3 is related to the linear viscoelastic modules (G' and G''), thereby supporting the hypothesis that TFF3 influence the viscoelastic properties of mucus. TFF1 has previously been shown to interact with mucin protein type 2 (MUC2)(10;22) and though the biochemical background for TFF3s influence remains to be elucidated, an

interaction between TFF3 and the mucins previously detected in the CMP (MUC1, MUC2, MUC5A and MUC5B) is most likely.

TFFs are present in virtually all mucus secreting epithelia and are often co-localized with specific mucins. TFF1 and TFF2 are present in the upper intestinal tract, whereas TFF3 is present mainly in the epithelia of the urogenital organs, lungs, and colon(7). Cervical mucus from non-pregnant women contains low concentrations of TFF1 (5.4 nM) and TFF2 (1.4 nM) but high concentrations of TFF3 (870 nM) (23). All three TFFs peak in concentration at ovulation (23) where mucus is thin, runny and shows “spinnbarkeit” (24;25). Even though proper rheological analyses of cervical mucus from non-pregnant women have never been conducted, the different viscoelastic characteristics of the mucus identified in these women may also be related to the observed changes of TFF1-3 content.

Based on the findings in this study, and in the studies referred to above, we find it most likely that TFF3 and perhaps also other TFFs, affect the viscoelastic properties of the CMP, thereby maintaining the ability of the CMP to protect against ascending infections from the vagina to the uterine cavity (5;9;26), yet the exact nature of TFFs interaction with mucins is still to be fully explained.

Acknowledgements:

We acknowledge Inger Marie Jensen, Department of Clinical Biochemistry, Aarhus University Hospital, for conducting the ELISA analysis of TFF1-3 and Kirsten Aggerholm Zeeberg, Research laboratory of Obstetrics and Gynecology, Aarhus University Hospital, for assisting with the extractions of the CMP biopsies. Also, we acknowledge Professor Michael Væth, Department of Biostatistics, Aarhus University for his assistance with the statistical analysis.

Funding:

This investigation was supported by grants from the The AP Møller Foundation for the Advancement of Medical Science, Direktør Jacob Madsens og Hustru Olga Madsens Fond, Familien Hede Nielsens Fond, and the Faculty of, Health Sciences Aarhus University.

Reference List

- (1) Becher N, Waldorf KA, Hein M, Uldbjerg N. The cervical mucus plug: structured review of the literature. *Acta Obstet Gynecol Scand.* 2009;88(5):502-13.
- (2) Hein M, Helmig RB, Schonheyder HC, Ganz T, Uldbjerg N. An in vitro study of antibacterial properties of the cervical mucus plug in pregnancy. *Am J Obstet Gynecol.* 2001;185(3):586-92.
- (3) Hein M, Valore EV, Helmig RB, Uldbjerg N, Ganz T. Antimicrobial factors in the cervical mucus plug. *Am J Obstet Gynecol.* 2002;187(1):137-44.
- (4) Hein M, Petersen AC, Helmig RB, Uldbjerg N, Reinholdt J. Immunoglobulin levels and phagocytes in the cervical mucus plug at term of pregnancy. *Acta Obstet Gynecol Scand.* 2005;84(8):734-42.
- (5) Boukari H, Brichacek B, Stratton P, Mahoney SF, Lifson JD, Margolis L, et al. Movements of HIV-virions in human cervical mucus. *Biomacromolecules* 2009;10(9):2482-8.
- (6) Chretien FC, Engelmann P, Dubois R. The variation of the mean spinability of human cervical mucus throughout the various stages of reproductive life. Automatic measurement and statistical study. *Eur J Obstet Gynecol Reprod Biol.* 1979;9(5):289-97.
- (7) Kjellek S. The trefoil factor family - small peptides with multiple functionalities. *Cell Mol Life Sci* 2009;66(8):1350-69.

- Accepted Article
- (8) Wiede A, Hinz M, Canzler E, Franke K, Quednow C, Hoffmann W. Synthesis and localization of the mucin-associated TFF-peptides in the human uterus. *Cell Tissue Res* 2001;303(1):109-15.
 - (9) Kindon H, Pothoulakis C, Thim L, Lynch-Devaney K, Podolsky. Trefoil peptide protection of intestinal epithelial barrier function: cooperative interaction with mucin glycoprotein. *Gastroenterology* 1995;109(2):516-23.
 - (10) Tomasetto C, Masson R, Linares JL, Wendling C, Lefebvre O, Chenard MP, et al. pS2/TFF1 interacts directly with the VWFC cysteine-rich domains of mucins. *Gastroenterology* 2000;118(1):70-80.
 - (11) Thim L, Madsen F, Poulsen SS. Effect of trefoil factors on the viscoelastic properties of mucus gels. *Eur J Clin Invest* 2002;32(7):519-27.
 - (12) Kjellek S, Nexø E, Thim L, Poulsen SS. Systemically administered trefoil factors are secreted into the gastric lumen and increase the viscosity of gastric contents. *Br J Pharmacol* 2006;149(1):92-9.
 - (13) Bastholm SK, Becher N, Stubbe PR, Chronakis IS, Uldbjerg N. The viscoelastic properties of the cervical mucus plug. *Acta Obstet Gynecol Scand* 2014;93(2):201-8.
 - (14) Barnes HA. A handbook of elementary rheology. Aberystwyth: University of Wales; 2000.
 - (15) Benham PP, Crawford RJ. Mechanics of engineering materials. Essex: Longman Scientific and Technical; 1987.
 - (16) Schramm G. A practical approach to rheology and rheometry. Karlsruhe: Gebrüder Haake; 1994.
 - (17) Steffe JF. Rheological methods in food process engineering. 2. ed. ed. East Lansing, Mich.: Freeman Press; 1996.

- Accepted Article
- (18) Becher N, Hein M, Danielsen CC, Uldbjerg N. Matrix metalloproteinases and their inhibitors in the cervical mucus plug at term of pregnancy. *Am J Obstet Gynecol* 2004;191(4):1232-9.
 - (19) Vestergaard EM, Poulsen SS, Gronbaek H, Larsen R, Nielsen AM, Ejekjaer K, et al. Development and evaluation of an ELISA for human trefoil factor 3. *Clin Chem* 2002;48(10):1689-95.
 - (20) Vestergaard EM, Brynskov J, Ejekjaer K, Clausen JT, Thim L, Nexø E, et al. Immunoassays of human trefoil factors 1 and 2: measured on serum from patients with inflammatory bowel disease. *Scand J Clin Lab Invest* 2004;64(2):146-56.
 - (21) Samson MH, Vestergaard EM, Milman N, Poulsen SS, Nexø E. Circulating serum trefoil factors increase dramatically during pregnancy. *Scand J Clin Lab Invest* 2008;68(5):369-74.
 - (22) Ruchaud-Sparagano MH, Westley BR, May FE. The trefoil protein TFF1 is bound to MUC5AC in human gastric mucosa. *Cell Mol Life Sci*. 2004;61(15):1946-54.
 - (23) Samson MH, Chaiyarit P, Nortvig H, Vestergaard EM, Ernst E, Nexø E. Trefoil factor family peptides in human saliva and cyclical cervical mucus. Method evaluation and results on healthy individuals. *Clin Chem Lab Med* 2011;49(5):861-8.
 - (24) Litt M, Wolf DP, Khan MA. Functional aspects of mucus rheology. *Adv Exp Med Biol* 1977;89:191-201.
 - (25) Elstein M. Functions and physical properties of mucus in the female genital tract. *Br Med Bull* 1978;34(1):83-8.
 - (26) Habte HH, de BC, Lotz ZE, Tyler MG, Schoeman L, Kahn D, et al. The inhibition of the Human Immunodeficiency Virus type 1 activity by crude and purified human pregnancy plug mucus and mucins in an inhibition assay. *Virol J* 2008;5:59.

Legends

Figure 1A-B. Representative plot of the elastic (black) and the viscous (grey) modulus obtained from **A.** Frequency Sweep and **B.** Stress Sweep (Black Arrow: Cross over point, Curly Bracket: Linear Viscoelastic Region). G' , Elastic Modulus; G'' , Viscous Modulus.

Figure 2. Dot plot showing concentrations of TFF1, TFF2 and TFF3 in cervical mucus plugs (CMP) removed at term from 14 women. (Hollow circle corresponding to the concentration for each CMP specimen, Gray dashed line: median TFF concentrations).

Figure 3A-D. The association between viscoelastic modules obtained by frequency sweep (at 0.01 Hz) and TFF3 (**A** and **B**). Association between viscoelastic modules obtained by stress sweep (at 1 Pa) and TFF3 (**C** and **D**). The 33 specimens (hollow circle) were obtained from cervical mucus plugs (CMP) retrieved at labor from 14 women. The regression line (black line) and 95% CI (gray dashed line) is based on all 33 samples and does not take the clustering of data account, this is dealt with in Table 2. G' , Elastic Modulus; G'' , Viscous Modulus.

Table 1. Mixed-effects restricted maximum likelihood regression was used to estimate the variation within and between cervical mucus plugs regarding the TFF1-3 concentrations. The statistical analysis is based on 33 specimens representing cervical mucus plugs removed from 14 women at labor. TFF measurements were log-transformed prior to analysis.

The TFF1-3 variation within and among CMPs			
		ICC (95% CI) ^a	<i>p</i> ^b
Trefoil factor peptide			
	TFF 1	0.87 (0.69-0.95)	< 0.001
	TFF 2	0.71 (0.34-0.92)	0.002
	TFF 3	0.87(0.71-0.96)	< 0.001

CMPs, cervical mucus plugs; ICC, intraclass correlation coefficients; CI, confidence interval.

^aICC (95% CI) gives the proportion of the residual variation attributable to between CMP variation.

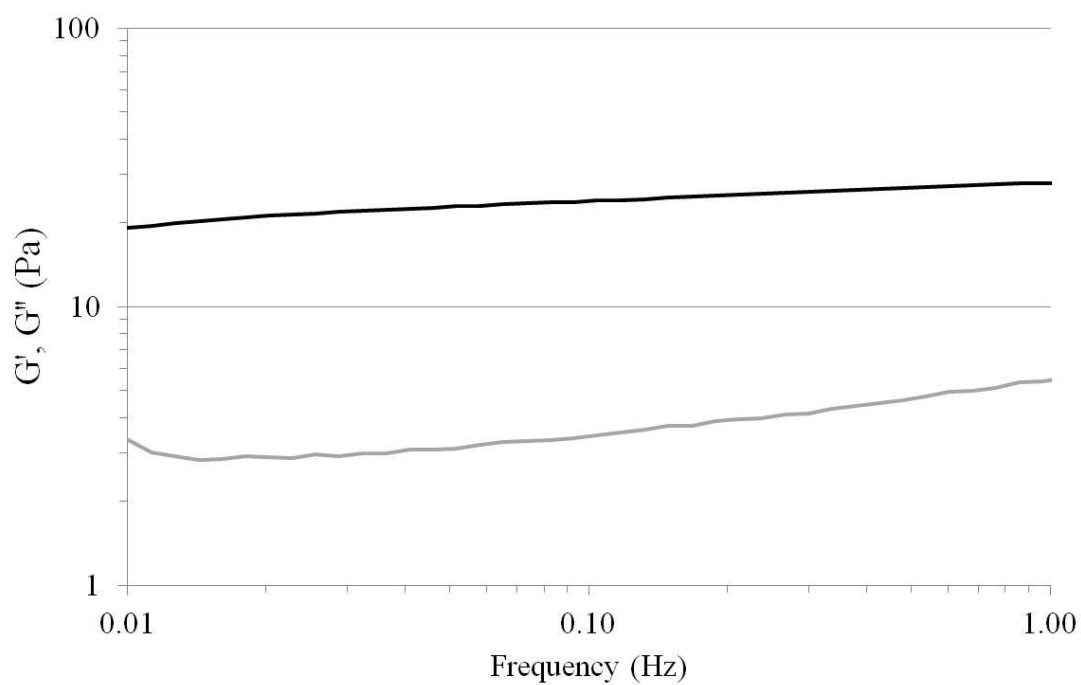
^b Likelihood ratio test (testing the hypothesis: between CMP variance = 0).

Table 2. Random-effects generalized least squares regression analysis was used to estimate the association between TFF1-3 concentrations and rheological variables obtained from frequency sweeps and stress sweeps. The statistical analysis is based on 33 specimens from cervical mucus plugs removed from 14 women at labor. TFF measurements were log-transformed in order to obtain a normal distribution and an equal variance. Shown is the TFF3 results (n = 33). Estimated regression coefficients (Reg. Coef.), 95% confidence interval (95% CI), two-sided *p*-values (*p*).

Association between TFF3 and rheological variables				
		Reg. Coef.	95% CI	<i>p</i>
Frequency sweep				
Elastic Modulus				
	0.01 Hz	11.7 Pa/Log nM	3.0 – 20.4	0.009
	0.1 Hz	13.1 Pa/Log nM	2.3 – 23.9	0.017
	1 Hz	15.5 Pa/Log nM	2.4 – 28.6	0.02
Viscous Modulus				
	0.01 Hz	3.2 Pa/Log nM	1.5 – 5.0	< 0.001
	0.1Hz	2.5 Pa/Log nM	0.8 – 4.1	0.004
	1 Hz	2.6 Pa/Log nM	0.2 – 5.0	0.035
Stress sweep				
Cross over point				
	Pa	-11.1 Pa/Log nM	-41.7 – 19.4	0.48
Elastic Modulus				
	1 Pa	15.1 Pa/Log nM	2.2 – 27.9	0.02
	15 Pa	14.4 Pa/Log nM	-0.4 – 29.2	0.06
	50 Pa	8.7 Pa/Log nM	-4.0 – 21.3	0.18
Viscous Modulus				

	1 Pa	2.5 Pa/Log nM	0.08 – 5.0	0.04
	15 Pa	2.5 Pa/Log nM	-0.35 – 5.3	0.09
	50 Pa	2.3 Pa/Log nM	-0.82 – 5.3	0.15

1A.



1B.

